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A Comparative *in vitro* Appraisal of Antioxidant Activity, Lipase Inhibition, Antidiabetic and Anticancer Effects of Fermented Milks Prepared using Different Lactic Strains

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ABSTRACT: The study evaluated fermented milks prepared using *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* BM24, highlighting their antioxidant, lipase-inhibitory, antidiabetic, and anticancer activities. Results showed strain- and time-dependent variations in biofunctional properties. All strains showed a consistant decline in pH during fermentation, with I4 exhibiting the lowest pH, indicating vigorous acid production. Antioxidant activity increased with fermentation time. BM24 showed the highest activity after 12 and 24 hours, followed by I4 and V3. At 24 hours, V3 exhibited the strongest lipase inhibition (80.35%), followed by I4 (70.87%) and BM24 (64.00%), suggesting potential for managing lipid digestion. V3 demonstrated the highest α-amylase inhibition (80.08%), while I4 showed the strongest α-glucosidase inhibition (57.73%). Against MCF-7 breast cancer cells, I4 exhibited the lowest IC50 value (36.26 µL at 24 hours), indicating the strongest anticancer potential. DAPI staining confirmed apoptotic features in treated cells, supporting the anticancer activity of fermentation-derived metabolites. These findings highlight the potential health benefitting properties of fermented milks of above-mentioned strains. Further the results underline the importance of strain selection and fermentation time for maximizing health benefits.

Keywords: Cell free supernatant, Biofunctional, Lactic acid bacteria, Apoptotic.

INTRODUCTION

Lactic acid bacteria (LAB), including species like *Lactobacillus*, play a crucial role in supporting a balanced and healthy human microbiota. These beneficial microorganisms have attracted considerable attention for their wide-ranging probiotic properties, which have been linked to numerous health benefits in both humans and animals (Latif *et al.*, 2023). A growing body of research indicates their role as protective agents against a broad array of diseases (Sreeja and Prajapati 2013; Latif *et al.*, 2023).

Fermented dairy products, particularly those produced with lactic acid bacteria (LAB), are renowned not only for their nutritional value but also for their diverse bioactive properties. Research studies emphasize the health benefits of fermented milk to the metabolic byproducts of fermentation and the specific traits of the microbial strains used. Different LAB strains uniquely enhance the functional properties of fermented milk, contributing to its antioxidant, antidiabetic, lipase inhibitory, and anticancer activities. These biofunctional attributes position fermented milk as a promising option for preventing and managing chronic conditions such as diabetes, obesity, and cancer (Shaikh and Sreeja 2017; Abdul Hakim *et al.*, 2023).

The inhibition of lipase activity is a significant factor in addressing metabolic diseases such as obesity. Lipases, which are enzymes responsible for lipid breakdown, play a key role in fat absorption. Their inhibition can reduce fat uptake and help manage body weight. Studies have demonstrated that certain LAB strains produce compounds capable of inhibiting lipase activity, thereby aiding in weight management and improving lipid profiles (Ménard *et al.*, 2017). As a result, fermented milk produced using specific LAB strains emerges as a promising functional food for obesity control and metabolic health improvement.

The antidiabetic properties of fermented milk have garnered increasing attention in recent years. Research has shown that bioactive components in fermented dairy products, including peptides, organic acids, and probiotic microorganisms, can positively affect glucose metabolism and improve insulin sensitivity. Notably, the fermentation process boosts the production of bioactive peptides with insulin-like effects, offering potential benefits for managing type 2 diabetes (Fu *et al.*, 2018; Lee *et al.*, 2019). Additionally, certain LAB strains have demonstrated the capacity to modulate

blood sugar levels, underscoring their therapeutic potential for individuals with diabetes.

Synthetic anticancer and antioxidative agents, while effective, are often associated with challenges such as high costs, side effects, and issues with long-term stability. In contrast, LAB and fermented dairy products have gained recognition as natural alternatives, with numerous studies highlighting their potential to reduce cancer risk and inhibit tumor progression (Biffi *et al.*, 1997; Lee *et al*., 2004). The anticancer properties of fermented milk are particularly noteworthy for their ability to influence pathways involved in cancer cell growth and apoptosis. Research indicates that certain LAB strains produce bioactive metabolites that can inhibit cancer cell proliferation, induce apoptosis, and prevent metastasis (Li *et al.*, 2013).

Beyond their anticancer potential, LAB are also celebrated for their antioxidative properties, which help combat oxidative stress—a key contributor to chronic diseases such as cancer and diabetes (Carini *et al.*, 2017; Poprac *et al*., 2017). Research has demonstrated the ability of certain LAB strains, including *Lactobacillus* and *Bifidobacterium*, to reduce oxidative stress effectively (Lin and Chang 2000; Kullisaar *et al.*, 2002). These findings underscore the therapeutic potential of LAB in managing oxidative stress-related conditions.

Given the wide range of bioactive properties associated with fermented milks, this study focuses on evaluating the antioxidant potential, lipase inhibitory activity, antidiabetic and anticancer effects of fermented milks prepared using different LAB strains. By examining the influence of various lactic strains on the functional attributes of fermented dairy products, this research aims to shed light on the potential health benefits of fermented milks and its role in the prevention and management of diseases.

MATERIALS AND METHODS

Lactic Strains, fermented milks. The lactic strains [*Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* BM24] were sourced from the culture collection of the Dairy Microbiology Department, SMC College of Dairy Science, Kamdhenu University, Anand, Gujarat, India. The strains are found to possess probiotic potential (results not part of this manuscript, Prajapati *et al*., 2011). For the preparation of fermented milk samples, these strains were inoculated in sterilized reconstituted skim milk with 12% total solids (TS) @ 2% and incubated at 37 ± 1°C for 12 and 24 hours. After incubation, the fermented milk samples were centrifuged at 10,000 rpm for 30 minutes at 4°C using an Eppendorf Centrifuge. The resulting supernatants were filtered through a 0.22 µm membrane filter to obtain Cell Free Supernatant (CFS). These fermented milk CFS were used for further analysis.

pH. The pH of the samples was determined using a pH meter (Oaklon pH 700 Benchtop Meter with Probe and Stand).

Antioxidant activity. Antioxidant activity was assessed using the ABTS [(2, 2'-Azino-bis (3-ethylbenzothiazoline 6-Sulphonic acid), Sigma-Aldrich, Bangalore, India] method, based on the protocol of Re et al. (1999) with some modifications. Total radical scavenging capacity was based on the ability of a compound to scavenge the stable ABTS radical in 10 min with some modifications. The water-soluble extracts of culture are used. The ABTS working solution was prepared by mixing 88 µl of 140 mM potassium per-sulphate with 5 ml of 7 mM ABTS stock solution and incubating overnight in dark bottles for generation of radicals. Then it was diluted with phosphate buffer saline to adjust the absorbance at 734 nm to 0.7 ± 0.02. An aliquot of 200 µl of culture supernatant, collected after centrifuging at 14,000 rpm for 30 min was mixed with 2300 µl ABTS and made up to volume 2500 µl. The decrease in absorbance at 734 nm was recorded over period of 10 min at 30 sec interval using spectrophotometer (systronics pc based double beam spectrophotometer 2206, India). Percent inhibition of absorbance at 734 nm was calculated using the following formula,

$$\% \text{ Radical scavenging activity} = \frac{(\text{A control} - \text{A test})}{(\text{A control})} \times 100$$

Where, A control = Absorbance of phosphate buffer solution at 734 nm

A Test = Absorbance of supernatant at 734 nm

Lipase Inhibition Activity. The lipase inhibitory activity was assessed following the procedure outlined by Gil-Rodríguez & Beresford (2020). Briefly, 500 µL of the fermented milk sample was aseptically added to a test tube containing 2 mL of Tris-HCl buffer (0.1 M, pH 8.25-8.75), prepared by mixing Trizma base (0.1 M, pH 10.48) and Trizma HCl (0.1 M, pH 4.9). To this, 50 µL of 4-nitrophenyl octanoate (5 mM, prepared in dimethyl sulfoxide [DMSO]) was added, followed by 50 µL of lipase (5 mg/mL in Tris-HCl buffer). The mixture was vortexed for 2 minutes and then incubated in a water bath at 37°C for 30 minutes. After incubation, 1 mL of clarifying reagent for dairy products was added, and the solution was incubated again at 37°C for 3 minutes. The absorbance was measured at 412 nm using a Systronics PC-based double beam spectrophotometer 2206 (Ahmedabad, India) against a Blank (without lipase), Control (without sample), and non-fermented milk control (non-fermented milk instead of the sample). The lipase inhibitory activity was calculated as a percentage of the activity relative to the 100% activity control using the following formula,

$$\text{Lipase inhibition } (\%) = 100 - \frac{(\text{Absorbance of sample-Absorbance of blank})}{(\text{Absorbance of the 100 \% activity control-Absorbance of the blank of this control})} \times 100$$

α-Amylase Inhibition Activity. α-Amylase inhibition was assessed according to the method described by Vankudre *et al.* (2015). To test tubes containing 300 µL of sample extract, 70 µL of 50% methanol, 50 µL of enzyme solution, and 1 mL of starch solution were added and incubated at 37°C for 5 minutes. Then, 2 mL

of 3, 5-Dinitrosalicylic Acid (DNSA) reagent was added, and the tubes were heated in a boiling water bath for 5 minutes, followed by cooling to room temperature. The absorbance of the resulting color was measured at 540 nm. Blank and control tubes, which contained no enzyme or sample, were prepared in parallel. α-Amylase inhibition was calculated as follows,

$$\text{Inhibitory activity (\%)} = 1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

α-Glucosidase Inhibitory Activity. α-glucosidase inhibition assay was conducted following the method of Apostolidis *et al.* (2006). Sample extract (500 µL) was incubated with 1000 µL of 0.1 M potassium phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/mL) at 25°C for 10 minutes. Then, 500 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in the same buffer was added, and the mixture was incubated for an additional 5 minutes. Absorbance was measured at 405 nm, with a control (buffer instead of sample) included. Acarbose (1 mg/mL) was used as a positive control, and non-fermented milk served as a negative control. α-Glucosidase inhibition was expressed as percentage inhibition.

The percentage α-Glucosidase inhibition activity was found using the formula;

$$\text{Inhibitory activity (\%)} = 1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Lactic count. Eleven grams of fermented milk was aseptically transferred to 99 mL of sterile phosphate buffer to create a 1:10 dilution. From this dilution, 1 mL was used to prepare subsequent dilutions in 9 mL phosphate buffer tubes. Appropriate dilutions were selected and pour plated in duplicate by adding 1 mL of the chosen dilution to sterile Petri dishes. These were mixed with 15–20 mL of sterile, cooled MRS agar. After the agar solidified, an additional layer of 5–7 mL of the same medium was added. The plates were incubated at 37 ± 1°C for 48 hours. Following incubation, typical Lactobacilli colonies were counted, and the results were expressed as log CFU/g.

MTT Assay. The MCF-7 cell line was sourced from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM supplemented with 10% FBS and maintained at 37°C in a 5% CO_2 incubator. The cytotoxicity of fermented milks against MCF-7 cells was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. For the experiment, 2×10^4 cells per well were seeded into 96-well plates and incubated overnight at 37°C in a CO_2 incubator. Cells were then treated with varying concentrations of CFS (25, 50, 75, 100, 150, and 200 µl) in triplicate, with the total volume in each well adjusted to 300 µl using the medium. Untreated wells served as controls. Following a 24-hour incubation at 37°C in a CO_2 incubator, MTT reagent was added to each well, and the plates were incubated for an additional 3 hours under the same conditions. The MTT dye was subsequently removed, and the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using an ELISA reader (TECAN, Switzerland), and the cytotoxic effect of CFS was expressed as the IC50 value. The IC50 value represents the concentration of the treatment required to achieve a 50% reduction in cell growth compared to the control cells.

DAPI Staining. The apoptosis-inducing effect of the CFS was evaluated using DAPI (4′,6-diamidino-2-phenylindole) staining, as described by Rezaei *et al.* (2012). In brief, cells (2×10^4 per well) were seeded into 6-well plates and treated with the IC50 concentration of the bacterial supernatant for 24 and 48 hours. Following treatment, the cells were fixed with methanol and stained with a 0.02% DAPI solution. The stained cells were then observed, and images were captured using a fluorescent microscope (Zeiss, AXI vert A1.F.L) equipped with a DAPI filter.

Statistical Analysis. The data related to the study were analysed using statistical design, Completely Randomized Design (CRD) and Factorial CRD using the software Web Agri Stat Package (WASP) developed by Indian Council of Agricultural Research (ICAR). Level of significance was kept at 5%.

RESULTS AND DISCUSSION

pH of the fermented milks prepared using *Lactobacillus rhamnosus* MTCC 5462 (I4), *Lactobacillus helveticus* MTCC 5463 (V3), and *Lactobacillus fermentum* BM24, were measured at 12 and 24 hours of incubation at 37°C. After 12 hours, the pH were 3.96, 4.46, and 4.74 respectively for *L. rhamnosus* (I4), *L. helveticus* (V3), and *L. fermentum* BM24. By 24 hours, the pH values had decreased to 3.35, 3.75, and 3.50 for the respective strains, indicating a consistent decline in pH over time (Table 1).

Table 1: Changes in the pH of fermented milks after 12 h and 24 h of incubation.

LAB strains	pH	
	12 h	24 h
Lactobacillus helveticus MTCC 5463(V_3)	4.46±0.019	3.75± 0.17
Lacticaseibacillus rhamnosus MTCC 5462(I4)	3.96± 0.049	3.35± 0.10
Limosilactobacillus fermentum BM24	4.74± 0.11	3.5± 0.08

The data represent mean values and standard deviations obtained from five replicate experiments.

The observed decrease in pH during the fermentation process suggest robust acid production by the LAB strains, a typical characteristic of lactic acid bacteria during milk fermentation. The strain-specific differences in pH are likely attributable to variations in the metabolic pathways utilized by each strain during fermentation. In particular, *L. rhamnosus* (I4) exhibited the lowest pH, which may be linked to its more vigorous fermentation activity compared to *L. helveticus* (V3) and *L. fermentum* BM24.

The antioxidant activity, measured by the ABTS+ scavenging effect, varied significantly among strains and incubation periods (Table 2). Results showed that antioxidant activity increased with incubation time. At 12 hours, CFS of *L. fermentum* BM24 exhibited the highest ($p<0.05$) activity (57.21%), followed by *L. rhamnosus* MTCC 5462 (36.05 %) and *L. helveticus* MTCC 5463 at (28.14 %). After 24 hours, the activity was highest ($p<0.05$) in *L. fermentum* (72.67 %), with *L. rhamnosus* and *L. helveticus* showing 50.21 % and 50.02 % respectively. The observed increase in antioxidant activity may result from extended fermentation, allowing for the accumulation of bioactive metabolites, such as peptides, organic acids and others. Prolonged fermentation promotes the biosynthesis of various metabolites like EPS and peptides, which significantly contribute to antioxidant activity (Song *et al.*, 2022; Zhou *et al.*, 2023).

Table 2: Antioxidant activity of CFS of fermented milks.

Incubation Period (h)	Antioxidant Activity (% radical scavenging)		
	V3	I4	BM24
12	28.14 ± 1.64^{aA}	36.05 ± 2.62^{aB}	57.21 ± 2.29^{aC}
24	50.02 ± 1.45^{bA}	50.21 ± 1.81^{bA}	72.67 ± 3.12^{bB}

The data represent mean values and standard deviations obtained from five replicate experiments. V3 = *Lactobacillus helveticus* MTCC 5463, I4 = *Lacticaseibacillus rhamnosus* MTCC 5462, BM24 = *Limocilactobacillus fermentum* BM24. Different uppercase alphabets as superscripts indicate significant difference among strains at specific interval of incubation at 5% level of significance and different lowercase alphabets as superscripts indicate significant difference among different interval of incubation time for a specific strain at 5% level of significance. Both incubation time and type of strain had significant ($p<0.05$) effect on the lipase inhibition activity of fermented milks (Table 3). After 12 hours, CFS of *L. helveticus* MTCC 5463 exhibited the highest ($p<0.05$) inhibition (58.14 %) followed by *L. fermentum* BM24 (45.55 %) and *L. rhamnosus* MTCC 5462(45.05 %). At 24 hours, MTCC 5463again demonstrated the highest ($p<0.05$) inhibition (80.35 %) followed by MTCC 5462 (70.87 %) and BM24 (64.00 %). The increase in inhibition from 12 to 24 hours highlights the critical role of incubation time in maximizing lipase inhibition. CFS of all the fermented milks showed promising (>50%) lipase inhibition after 24 h of incubation. Among the fermented milks, CFS of *L. helveticus* MTCC 5463(V3) showed the most significant inhibition at both time points, suggesting it produces bioactive compounds with strong lipase-inhibitory properties, making it a promising candidate for managing lipid digestion and absorption. Moderate inhibition levels in CFS of *L. rhamnosus* (I4) and *L. fermentum* BM24 indicate these strains also generate lipase inhibitors, though to a lesser extent. These results align with previous studies identifying *L. helveticus* as a producer of antimicrobial and enzyme-inhibiting metabolites, particularly against digestive enzymes such as lipases (Zhao *et al.*, 2020; Li *et al.*, 2021).

The antidiabetic potential of fermented milks was evaluated by assessing α-amylase and α-glucosidase inhibitory activities. CFS of *L. helveticus* MTCC 5463 (V3) demonstrated the highest ($p<0.05$) α-amylase inhibitory activity, with values of 76.51% at 12 hours and 80.08% at 24 hours, followed by *Lactobacillus fermentum* BM24 (75% and 75.53%) and *Lactobacillus rhamnosus* (I4) (69.69% and 74.40%). The results indicated no significant differences in α-amylase inhibition between incubation times or strains for *L. helveticus* (V3) and *L. rhamnosus* (I4). For α-glucosidase inhibition, *L. rhamnosus* (I4) exhibited the highest activity ($p<0.05$), with values of 51.69% at 12 hours and 57.73% at 24 hours. In contrast, *L. fermentum* BM24 and *L. helveticus* (V3) showed lower inhibition levels at both time points. Notably, the strong α-amylase inhibition by *L. helveticus* (V3) and high α-glucosidase inhibition by *L. rhamnosus* (I4) suggest their effectiveness in controlling postprandial blood glucose levels. The observed inhibitory effects may be attributed to bioactive peptides produced during fermentation, which are known to modulate carbohydrate digestion and absorption. Additionally, *L. helveticus* has been reported to generate peptides with ACE-inhibitory and antidiabetic properties (Huang *et al.*, 2020). Previous studies also support the role of probiotics and fermented foods in modulating gut microbiota and improving insulin sensitivity (Khatri *et al.*, 2021; Zhang *et al.*, 2023).

Table 3: Lipase inhibitory activity of CFS of fermented milks.

Incubation Period (h)	Inhibition (%)		
	V3	I4	BM24
12	58.14 ± 1.64^{aB}	45.05 ± 1.53^{aA}	45.55 ± 2.60^{aA}
24	80.35 ± 1.62^{bC}	70.87 ± 2.20^{bB}	64 ± 1.82^{bA}

The data represent mean values and standard deviations obtained from five replicate experiments. V3 = *Lactobacillus helveticus* MTCC 5463, I4 = *Lacticaseibacillus rhamnosus* MTCC 5462, BM24 = *Limocilactobacillus fermentum* BM24. Different uppercase alphabets as superscripts indicate significant difference among strains at specific interval of incubation at 5% level of significance and different lowercase alphabets as superscripts indicate significant difference among different interval of incubation time for a specific strain at 5% level of significance.

The lactic counts in the fermented milks were determined by pour plate technique. After 12 hours, fermented milk of *L. helveticus* (V3) showed the

highest count at 10.16 log cfu/g, followed by *L. fermentum* BM24 (9.72 log cfu/g) and *L. rhamnosus* (I4) (9.48 log cfu/g). Similarly, after 24 hours, the counts in the fermented milk of *L. helveticus* (V3) reached 10.18 log cfu/g followed by *L. fermentum* BM24 at 9.69 log cfu/g, and *L. rhamnosus* (I4) at 10.04 log cfu/g. Probiotic viability, as indicated by colony counts, remained high (> log 9 CFU/g) in all fermented milks after 12 and 24 hours of incubation. This is critical for their application in functional food products, as high viable counts are necessary to confer health benefits.

Table 4: Alpha amylase inhibition of CFS of fermented milks.

Incubation Period (h)	Alpha amylase inhibition (%)		
	V3	I4	BM24
12	76.51 ± 2.13^{bA}	69.69 ± 2.59^{aA}	75.00 ± 2.99^{bA}
24	80.08 ± 2.75^{bB}	74.40 ± 1.86^{aB}	75.53 ± 1.90^{aA}

Table 5: α – glucosidase inhibition of CFS of fermented milks.

Incubation Period (h)	α - glucosidase inhibition		
	***L. helveticus* MTCC 5463(V_3)**	***L. rhamnosus* MTCC 5462 (I4)**	***L. fermentum* MW07743 (BM24)**
12 h	24.18 ± 2.70^{aA}	51.69 ± 2.25^{aA}	31.67 ± 2.93^{aA}
24 h	30.08 ± 2.75^{bA}	57.73 ± 5.26^{bB}	55.53 ± 1.90^{bB}

The data represent mean values and standard deviations obtained from five replicate experiments. V3 = *Lactobacillus helveticus* MTCC 5463, I4 = *Lacticaseibacillus rhamnosus* MTCC 5462, BM24 = *Limocilactobacillus fermentum* BM24. Different uppercase alphabets as superscripts indicate significant difference among strains at specific interval of incubation at 5% level of significance and different lowercase alphabets as superscripts indicate significant difference among different interval of incubation time for a specific strain at 5% level of significance.

In vitro cytotoxic activity of CFS derived from all the fermented milk samples (after 12 h and 24 h of incubation) were evaluated using MTT assay on breast cancer cell line MCF-7. The IC_{50} value representing the CFS's inhibitory concentration causing 50% cell population death was calculated based on the dose–response curve generated after obtaining percent cell death at different concentrations of the CFS. Cytotoxicity was expressed as the mean IC_{50} of three independent experiments. The IC_{50} values of CFS of V3, I4 and BM24 were in the range of 36.26 – 88.75. The study findings (Fig. 1) revealed a significant decrease in the IC_{50} values for the 24 hour CFS in contrast to the IC_{50} of the 12 hour CFS, indicating dose- and time-dependent cytotoxicity. The IC_{50} values of CFS (24 h) of V3, I4 and BM24 were 79.27 µL, 36.26 µL, 73.43 µL respectively. However, the values (88.75 µL for V3, 40.59 µL for I4 and 74.70 µL for BM24) were higher for CFS of all strains obtained from 12 h fermented milks. CFS(24 h) of *L. Rhamnosus* exhibited lowest IC_{50} value of 36.26. The findings suggest a notable variability in the antiproliferative effects of the CFS of probiotic strains, with *Lactobacillus rhamnosus* (I4) demonstrating the most potent activity against MCF-7 cells. These effects may be attributed to the specific production of microbial metabolites such as organic acids, bacteriocins, bioactive peptides, and others during fermentation (Lee *et al.,* 2018). This hypothesis is supported by studies on the impact of fermentation time on the stability and bioactivity of metabolites such as lactic acid and SCFAs, which have been reported to modulate cancer cell apoptosis (Kiousi *et al.,* 2023; Koeth *et al.,* 2016). Furthermore, the differential cytotoxicity observed in the present study may be strain-dependent, as different probiotic strains have been shown to possess distinct abilities to modulate immune responses and induce apoptosis in cancer cells (Zhao *et al.,* 2020).

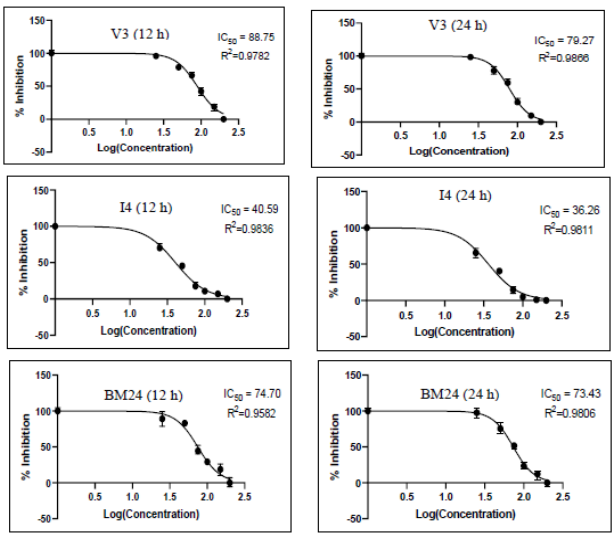

Fig. 1. Cytotoxic activity of CFS of fermented milks of different LAB strains *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus fermentum* (BM24).

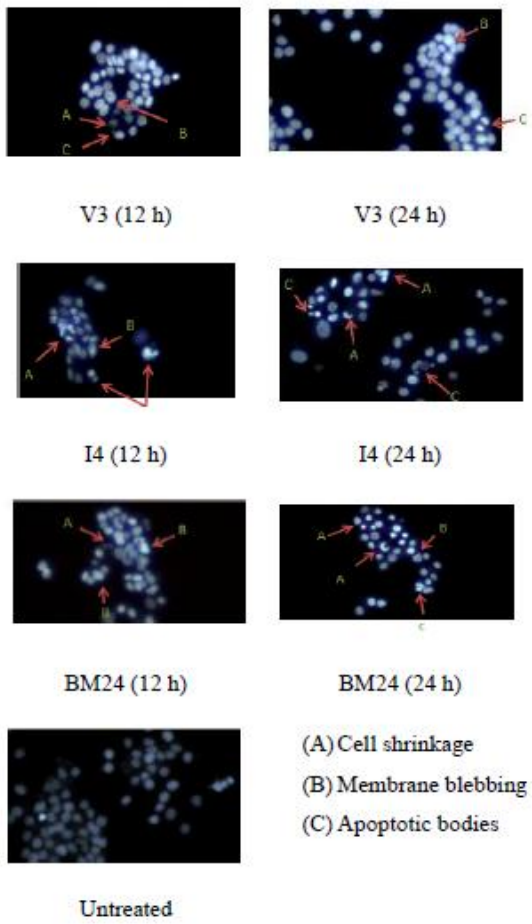

Fig. 2. Morphological changes in MCF-7 cells with and without CFS treatment after DAPI staining *Lactobacillus helveticus* MTCC 5463 (V3), *Lacticaseibacillus rhamnosus* MTCC 5462 (I4), and *Limosilactobacillus* fermentum (BM24).

Additionally, to further assure the apoptotic potential of the CFS, the MCF-7 cells were treated with DAPI, a cell-permeable fluorescence dye that strongly binds to A–T-rich regions of DNA and aids in analyzing nuclear condensation and fragmentation. After 48 hours of treatment at the respective IC_{50} doses, nuclear condensation and fragmentation were evident in the treated cells, along with other apoptotic features such as cell shrinkage and membrane blebbing (Fig. 2). Recent studies have identified that peptides generated through the fermentation process, particularly from milk proteins, possess bioactive properties that can inhibit cancer cell proliferation and induce apoptosis (Duarte *et al*., 2020; Ray and Nair 2022). These peptides, such as lactoferrin-derived peptides and casein fragments, have been reported to exhibit antioxidant, anti-inflammatory, and anticancer activities, thereby contributing to their cytotoxic effects (Raimondi *et al.*, 2018). The cytotoxicity assay, coupled with the morphological changes observed in the MCF-7 cells through DAPI staining, provides evidence that the probiotic samples possess anticancer activity against MCF-7 breast cancer cells.

CONCLUSIONS

This study highlights the potential of fermented milks of *Lactobacillus helveticus* MTCC 5463, *Lacticaseibacillus rhamnosus* MTCC 5462, and *Limosilactobacillus fermentum* BM24 for their antioxidant, lipase-inhibitory, antidiabetic, and anticancer activities. The findings demonstrate that these biofunctional properties are highly dependent on both the strain and the fermentation duration. Among the strains, *Lacticaseibacillus rhamnosus* MTCC 5462 exhibited the strongest cytotoxic potential, positioning it as a promising candidate for further exploration in anticancer applications. The strain-specific differences in bioactivity underline the need for detailed studies to uncover the underlying mechanisms. Given their significant biofunctional potential, these strains could be employed to develop functional fermented dairy foods offering substantial health benefits.

FUTURE SCOPE

Future research should focus on isolating and characterizing the bioactive compounds responsible for the bioactivities and investigating their mechanisms of action comprehensively. The findings of the current study should be validated through *in vivo* experiments using animal models.

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